

## New Plasmid System To Select for *Saccharomyces cerevisiae* Purine-Cytosine Permease Affinity Mutants

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**The *FCY2* gene of *Saccharomyces cerevisiae* encodes a purine-cytosine permease (PCP) that mediates the active transport of purines and cytosine. A structure-function model for this PCP has been recently proposed. In this study, we developed a plasmid-based system that generated a number of affinity-mutated alleles, enabling us to define new amino acids critical for permease function.**

**Design of a plasmid system for the selection of PCP affinity mutants.** In the yeast *Saccharomyces cerevisiae*, adenine (ADE), guanine (GUA), hypoxanthine (HYP), and cytosine (CYT) are taken up with the same affinity by a single proton symporter purine-CYT permease (PCP) encoded by the *FCY2* gene (4, 9, 12, 13). Disruption of this gene abolishes active transport, despite the presence in the yeast genome of *FCY21* and *FCY22* (accession number X97346), which belong to the same gene family. Several mutants with modified uptake abilities have been selected in vivo (2, 4, 6), and their analysis has led to the generation of a functional model for this permease (7).

To test this model and to investigate further the structure-function relationship of the PCP, we isolated additional *fcy2* affinity mutant alleles by various types of in vivo positive selection, making use of the broad specificity of this permease. PCP affinity mutants were isolated as previously described (4), by selecting cells able to overcome growth inhibition due to the outcompetition of two of the PCP substrates for entry into the cell. Two strains were constructed, RW123 ( $\Delta fcy2::HIS3 ade1-1 his3\Delta200 trp1\Delta$  [pPZ1-7 *FCY2 TRP1*]) and RW126 ( $\Delta fcy2::HIS3 ura2_{9-15-30} his3\Delta200 trp1\Delta$  [pPZ1-7 *FCY2 TRP1*]), in which the *FCY2* coding sequence was replaced with the *HIS3* marker as previously described (1) and the wild-type *FCY2* allele was carried on the pPZ1-7 single-copy plasmid (2). Both were used for selection with various pairs of substrates (Table 1). The presence of a plasmid-borne *FCY2* allele made it easier to clone and sequence the selected mutations.

Due to its *ade1-1* mutation, strain RW123 was auxotrophic for ADE and therefore required efficient ADE uptake by the PCP for growth. When this strain was plated on YNB medium containing a low concentration of ADE and a high concentration of another substrate of the PCP, the competition between the two bases prevented the uptake of sufficient quantities of

ADE, leading to growth inhibition. This growth inhibition was achieved by adding ADE at  $3 \mu\text{g} \cdot \text{ml}^{-1}$  to the medium along with CYT at  $400 \mu\text{g} \cdot \text{ml}^{-1}$  or 5-methylcytosine (5MeC) at  $200 \mu\text{g} \cdot \text{ml}^{-1}$  as a competitor. The use of these two competitors defined two selection schemes, facilitating the selection of CYT-resistant (CYT<sup>r</sup>) and 5MeC-resistant (5MeC<sup>r</sup>) mutants, respectively.

Strain RW126, which carried the *ura2\_{9-15-30}* mutant allele, required an exogenous pyrimidine source, such as CYT, for growth. Thus, CYT at  $3 \mu\text{g} \cdot \text{ml}^{-1}$  was added to the medium as the limiting substrate and the competitor was ADE, HYP, GUA, or 5MeC. Competition conditions were achieved at the following concentrations:  $150 \mu\text{g} \cdot \text{ml}^{-1}$  for ADE,  $150 \mu\text{g} \cdot \text{ml}^{-1}$  for HYP,  $60 \mu\text{g} \cdot \text{ml}^{-1}$  for GUA, and  $200 \mu\text{g} \cdot \text{ml}^{-1}$  for 5MeC. Thus, based on the use of these four competitors, we were able to define four selection schemes, facilitating the selection of ADE<sup>r</sup>, HYP<sup>r</sup>, GUA<sup>r</sup>, and 5MeC<sup>r</sup> mutants, respectively.

**Selection of affinity mutants.** Five hundred five independent mutants obtained from 821 rounds of selection were retained for further analysis. Fifty-eight contained a mutation in the plasmid-borne *FCY2* gene; 50 were selected by competition between ADE and CYT in strain RW123(pPZ1-7); and 8 were selected by competition between CYT and 5MeC in strain RW126(pPZ1-7). The remaining 447 mutants displayed chromosomal mutations. Four of the six selection systems used did not generate plasmid mutations (Table 1).

The sequences of the 58 plasmid-borne mutant *fcy2* alleles defined eight classes of mutations (Table 2). Five of these classes corresponded to single missense mutations (paC-A, paC-B, and pcM-A to -C), and three corresponded to extensive nucleotide replacements resulting in several amino acid changes (paC-C, pcM-D, and pcM-E). We assessed substrate uptake in the mutants to determine the kinetic parameters of transport  $V_{\text{max}}$  (maximal rate of uptake) and  $K_{\text{tapp}}$  (apparent Michaelis constant of transport) for the various substrates of the PCP (Table 2).

**Single missense mutations in the S369-to-N377 segment.** Four of the five single missense mutations affected a short peptide segment of the PCP, from serine 369 (S369) to asparagine 377 (N377), corresponding to a hydrophilic external loop of the PCP (7) (Fig. 1). Three of these mutations corresponded

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TABLE 1. Selection of PCP affinity mutants

Strain	Selective medium <sup>a</sup>		Mutant phenotype	No. of independent selections <sup>b</sup>	No. of analyzed mutants <sup>b</sup>	No. of plasmidic mutations <sup>b</sup>
	Competing substrate (concn, $\mu\text{g} \cdot \text{ml}^{-1}$ )	Limiting substrate (concn, $\mu\text{g} \cdot \text{ml}^{-1}$ )				
RW123(pPZ1-7)	CYT (400)	ADE (3)	CYT <sup>r</sup>	191	183	8
	5MeC (200)	ADE (3)	5MeC <sup>r</sup>	50	65	0
RW126(pPZ1-7)	ADE (100)	CYT (3)	ADE <sup>r</sup>	215	89	0
	HYP (100)	CYT (3)	HYP <sup>r</sup>	145	73	0
	GUA (60)	CYT (3)	GUA <sup>r</sup>	110	14	0
	5MeC (200)	CYT (3)	5MeC <sup>r</sup>	110	81	50
Total				821	505	58

<sup>a</sup> Compositions of YNB selective media and phenotypes of selected mutants.<sup>b</sup> Number of isolated mutants and characterization of *fcy2* mutations.

to amino acid substitutions not previously found (S369F, T370N, and N377T). For these three substitutions, the  $K_{\text{tapp}}$  for ADE was close to the value obtained with a wild-type strain but the  $K_{\text{tapp}}$  was significantly higher than that of the wild-type strain for CYT, HYP, and GUA.

In the T370N mutant, these changes were also accompanied by a general decrease in  $V_{\text{max}}$  values (by a minimum factor of 2 for ADE and a maximum of 20 for GUA), suggesting that the amino acid substitution also slowed down the overall transport process. In contrast, the N377I substitution obtained by this approach and in a previous study (2) led to a large increase in all  $K_{\text{tapp}}$  values (by factors of 13, 16, 10, and 17 for ADE, CYT, HYP, and GUA, respectively) and the  $V_{\text{max}}$  for CYT was also significantly higher than that of the wild type (about seven times as high).

These four point mutations in the S369-to-N377 segment accounted for more than 80% of the selected clones, indicating the important role played by this short peptide segment in the permease activity. These new results and the I371, N374, I375, P376, and N377 substitutions previously studied (2, 6, 8) clearly show that this region is involved in maintaining the PCP in a functional configuration, probably playing an active part in the conformational changes needed for solute translocation. In particular, asparagine 377 is thought to play an essential role in the solute-binding site, which is consistent with our results,

because more than 70% of the selected mutations (41 of 58) affected this residue.

**Modification of the G280 residue.** Replacement of the glycine at position 280 with an alanine (G280A) resulted in a global increase in  $K_{\text{tapp}}$  values for all substrates (by a factor of six to seven) and in a similar decrease in  $V_{\text{max}}$  values, except for CYT. These strong effects on global uptake parameters indicate that glycine 280 is involved in the transport process. Moreover, considering the weak structural change responsible for these strong effects (basically, replacement of a hydrogen atom with a methyl group), glycine 280 has to be strongly conserved for maintenance of PCP activity.

This residue is located in a hydrophilic external loop, adjacent to that containing the S369-to-N377 segment (7) (Fig. 1). It therefore seems likely that these two predicted loops are involved in the correct folding of the protein and/or the establishment of the active site. This is consistent with the results of a recent study (7) that identified residue S372, located in the same region as G280, as a potential intragenic suppressor of the N374I substitution.

**Multimutated alleles.** Three plasmid mutants showed multiple missense mutations in the *fcy2* sequence. The region mutated depended on the allele: 17 mutations led to four amino acid substitutions for the paC-C allele, 17 mutations led to three amino acid substitutions for pcM-D, and 37 mutations

TABLE 2. Amino acid substitutions and kinetic parameters ( $K_{\text{tapp}}$  and  $V_{\text{max}}$ ) of products of mutated *FCY2* alleles<sup>a</sup>

FCY2 allele	No. of selected alleles	Amino acid replacement(s)	Transport parameters							
			ADE		CYT		HYP		GUA	
			$K_{\text{tapp}}$	$V_{\text{max}}$	$K_{\text{tapp}}$	$V_{\text{max}}$	$K_{\text{tapp}}$	$V_{\text{max}}$	$K_{\text{tapp}}$	$V_{\text{max}}$
Wild type			1.2	175	2.3	240	2.5	200	1.6	220
paC-A	6	S369F	2.8	305	26	780	14	115	12.5	110
paC-B	1	T370N	1.4	86	18	59	16.5	12	9.4	11
pcM-A	31	N377I	16	248	36.5	1,750	25	437	28.2	99
pcM-B	10	N377T	0.67	58	9	453	14.1	147	20.3	57
pcM-C	7	G280A	9.5	35	13.5	380	13.2	33	13.5	64
paC-C	1	I371V, I375V, N377G, V478A	2.8	250	25	605	23.2	65	10	27
pcM-D	1	I371V, I375V, N377G	3.1	288	30	735	17.6	48	28.8	57
pcM-E	1	A335S, I371V, I375V, N377G, S441A, F447I	2.9	253	27.7	495	15.8	38	70	107

<sup>a</sup> paC-type mutated alleles are from CYT<sup>r</sup> mutants of strain RW123, and pcM-type alleles are from 5MeC<sup>r</sup> mutants of strain RW126.  $K_{\text{tapp}}$  values are apparent Michaelis constants of transport (micromolar).  $V_{\text{max}}$  values are maximal rates of uptake expressed in picomoles of incorporated substrate per  $10^7$  cells per minute.  $K_{\text{tapp}}$  and  $V_{\text{max}}$  values were determined as described by Br  thes et al. (3), with seven concentrations of radiolabeled substrate. Experiments were performed in duplicate.

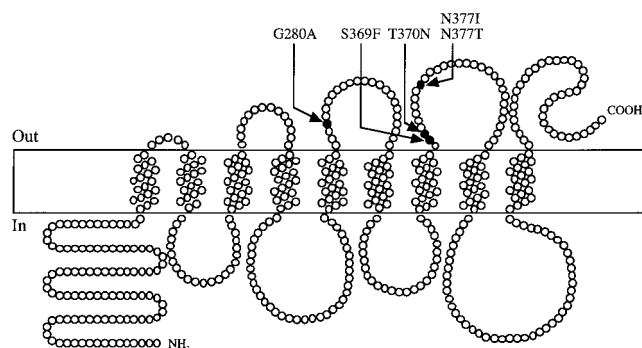


FIG. 1. Positions of selected amino acid substitutions in the PCP model proposed by Ferreira et al. (7).

led to six amino acid substitutions for pcM-E. These mutated regions displayed sequences identical to that of *FCY22*, strongly suggesting that ectopic recombination had occurred between the plasmid-borne *FCY2* allele and the *FCY22* gene. The recombinant sequences were 100 to 400 bp in size, and all surrounded the region encoding the hydrophilic S369-to-N377 segment. The three alleles had three substitutions in common: I371V, I375V, and N377G. Previous site-directed mutagenesis experiments (2) showed that the mutant phenotype resulted principally from the N377G substitution. As the *FCY21* gene encodes an asparagine residue at this position, we believe that recombination between this duplicated gene and *FCY2* may well occur but cannot result in the mutant phenotype.

The effects on substrate uptake parameters were similar in the three mutants: ADE incorporation was not affected, the  $K_{\text{tapp}}$  for CYT was about 10 times higher than that in the wild type, that for HYP was 6 to 9 times higher, and that for GUA was also significantly higher. However, the increase in  $K_{\text{tapp}}$  for GUA depended on the size of the mutated sequence, from a sixfold increase for paC-C to an increase of more than 40-fold for the largest mutated allele, pcM-E. This size-dependent modification indicated that one or more of the additional amino acid replacements was responsible for further functional changes in GUA uptake, suggesting that other features of the structure-function relationship are important.

**General considerations.** All of the mutations selected in this work and in previous studies affect hydrophilic segments predicted to be external or cytoplasmic; none affect hydrophobic regions. Consistent with recent studies of bacterial and fungal nucleobase transporter mutants with altered substrate specificity (5, 11), this strongly suggests that discrimination among the four substrates of the PCP probably occurs during the binding step, which is then followed by a common translocation process. Moreover, a global decrease in apparent affinity for CYT, GUA, and HYP was observed for all of the selected mutations, suggesting that the structural discriminants involved in ADE recognition differ from those for CYT, GUA, and HYP binding. Thus, every uptake modification for one of these three molecules should lead to a similar effect for the other two. This observation may account for the results of

some of the screenings, involving affinity decoupling between CYT and GUA, or between CYT and HYP, in which PCP mutants were not isolated in our selection scheme.

Only about 10% of the mutants selected with this approach bore an expected mutant plasmid-borne *fcy2* allele (58 of 505). Thus, most of the mutations selected corresponded to chromosomal loci. It would be of great interest to study these mutations, as they reflect a significant adaptive response to major substrate variations. For instance, some of these mutants may result from the functional modification of other transporters, as has been observed in similar studies: during selection for suppressors that restored potassium uptake in  $\Delta\text{trk1 } \Delta\text{trk2}$  cells, unusual gain-of-function mutations were characterized in various transporters, such as amino acid permeases Bap2 and Hip1, glucose transporters Hxt1 and Hxt3, and glucose-galactose transporter Gal2 (10, 14).

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